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13. ABSTRACT (Maximum 200 words) We have developed an approach to the identification of retroposed retroviral sequences in the human genome. This method uses nested PCR directed toward the LTRs of endogenous retroviruses, and arbitrary priming to complete the other end of the PCR product. Our results indicate that we can reliably identify sequences flanking retrovirus proviral sequences. This will allow us to probe BAC and YAC arrays with probes derived from tumors to determine whether the tumor displays the movement of a retrovirus between chromosomal positions. This array-based method will also lend itself to more facile examination of the products that result from RDA and SSH subtraction methods. Thus, we are now armed with an arsenal of tools for tracking retroviral retroposition events associated with human malignancy.				
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FOREWORD

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Introduction

In this progress report, we present evidence that we have developed a method that should be adequate for identification of retrotransposition events involving retroviral sequences. The following information from the previous progress report outlines our original reasoning.

HERVs which make up an estimated 1% of the entire human genome are grouped into single and multiple copy families. Although most of them seem to be defective due to multiple termination codons and deletions, some of them are full-length proviruses and transcriptionally active. HERV transcription may play a role during normal gene expression (some transcripts are very abundant in placenta) and it has been suggested that this role could be a protective function against superinfection by related exogenous retroviruses.

However, overexpression of HERVs has been reported in various cancer cell lines, such as teratocarcinoma cell lines and cell lines derived from testicular and lung tumors. Expression of HERVs was shown to be associated with mouse strains susceptibility to lung cancer. In our laboratory, we have found a variation in the expression of different family members of HERVs in some colorectal cancer cell lines. The implications of expression of HERV sequences in pathophysiological processes remains to be elucidated.

There is substantial evidence that HERVs are still retrotransposing. As the retroviral long terminal repeat (LTR) sequences of HERVs contain complex regulatory elements such as promoters, enhancers, transcription initiation sites and polyadenylation signals, their insertion in the vicinity of host gene could cause a dramatic change in its expression. The current project relies on the hypothesis that HERVs have a role as mutagenic agents in at least a subset of breast cancers. This research focuses on two different families of HERVs: HERV-H family, that is the most abundant (1000 copies in addition to a similar number of solitary LTRs), and HERV-K family, that is characterized as the most "active" family. We are looking for differential HERV reverse transcriptase activity in cancer lines in response to growth factor and hormone treatments as well as in fresh breast cancer tissues. We are trying to determine if integration of cDNA occurs at an enhanced rate in cancer cells by looking for new integration sites of HERV sequences in tumor tissues. Thus, the purpose of this study is to develop ways to identify new insertional events that might result in breast cancer, and to gather other evidence that retroposition of endogenous retroviruses is an active mechanism of cancer.

Body

Summary of earlier results

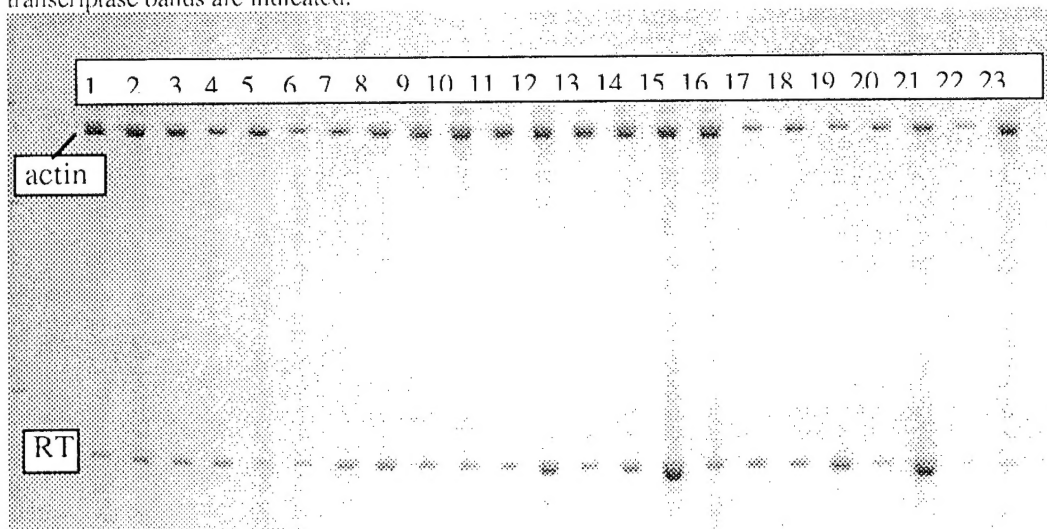
This section is synopsised from the previous annual report.

Elevation of Reverse Transcriptase Activity

We demonstrated several instances of elevated levels of reverse transcriptase have appeared in our screens of normal vs. tumor pairs, and in cancer-derived cell lines. These results were discussed in the previous annual report. Briefly, RNA was isolated from two normal cell lines, eight different cancer cell lines, and from normal and cancer tissues originating from anonymous patients. Reverse transcriptase activity was measured through the abundance of retroviral reverse transcriptase mRNAs (HERV-H family) in the different cell lines and normal-tumor pairs. Figure 1 shows the results of RT-PCR directed toward retroviral sequences for normal-tumor pairs, and for cancer derived cell types. Two tumors (lanes 19, 21) as well as two tumor derived cell lines (12, 15) show elevated levels of transcript for reverse transcriptase. This behavior is consistent with what would be expected for a retroposition mechanism.

Figure 1. Retroviral Reverse Transcriptase transcript overexpression in cell lines and tumors, analyzed by RT-PCR.

1-2, cancer cell lines; 3-8, normal-tumor pairs; 9-10, normal cell lines, 11-16 cancer cell lines; 17-20, normal-tumor pairs; 21, unpaired tumor; 22-23, normal-tumor pair. Actin control and retroviral reverse transcriptase bands are indicated.



PCR of flanking genomic sequences.

PCR was performed using a primer specific for the LTRs of members of the HERV-H family (RTVL-H2, RGH-1, RGH-2). Specific targeting of HERV LTRs was confirmed by the use of nested primers generating products 10 basepairs shorter (**Figure 2**). The first PCR step involved a primer specific for the LTRs of the members of the HERV-K family (K10, HML6), under low stringency conditions. The second primer was specific for the LTRs of the member of the HERV-H family (RTVL-H2, RGH-1, RGH-2). The PCR was run under high stringency conditions to ensure specific sampling of HERV-H LTRs. The protocol was elaborated using cancer cell lines, then five matching pairs of normal and tumor tissue DNAs were screened for differences between normal and tumor tissues. Only one difference was detected in these fingerprints.

Figure 2 contains LTR to arbitrary products for 5 normal-tumor pairs, with the original primer pair in the left panel and the nested pair in the right panel. Each sample is represented in two lanes, where input RNA concentrations were titrated by a factor of two. The effect observed (arrows point several of the many examples) indicates that the initial primer pair targets predominantly retroviral sequences in the genome, because nesting the primers with respect to the original primers according to known, conserved 3' sequence causes a 10 base pair shift consistent with the 10 base pair nesting from one end. Sequence analysis of several of these bands confirmed that these bands usually derive from an LTR on one end, and arbitrary priming at the other. This experiment raised the interesting possibility that genomic clone (BAC or YAC) arrays, which are now commercially available, could be used to discover new retroviral insertion sites. We have performed such a study since the last annual report, the results of which are introduced in the section entitled "Recent work".

Figure 2. Nested PCR shows that retroviral sequences are targeted.

Normal-tumor pairs. Left: A primer directed outward from the LTR was used in conjunction with arbitrary priming to amplify sequences flanking retroviral sequences in the genome. Right: nested primer secondary amplification of the right hand fingerprint. Lanes 1,3,5,7,9 = tumor; Lanes 2,4,6,8,10, normal.

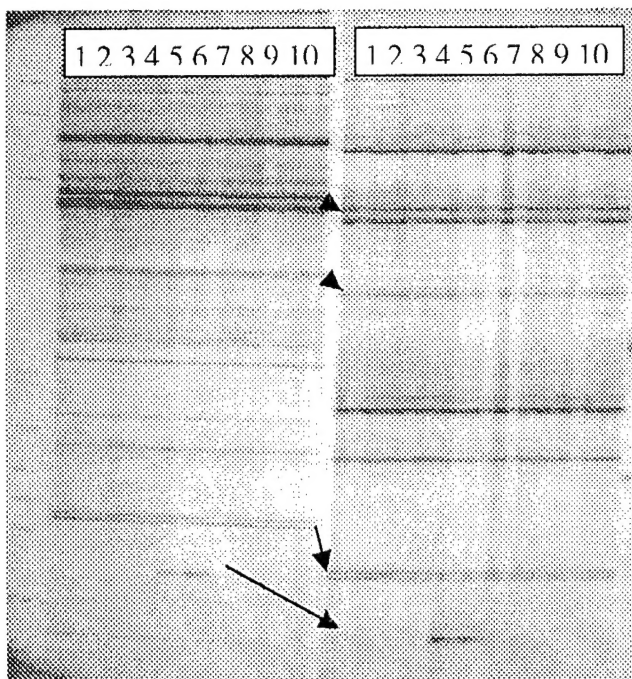
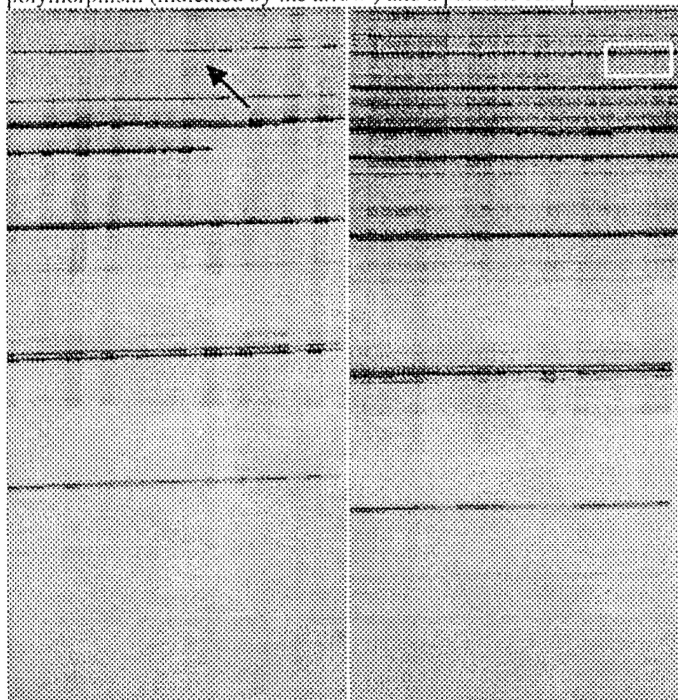


Figure 3. Additional example of LTR to Arbitrary sampling of retroviral flanking sequences.

This example also contains normal-tumor pairs, shows the shift due to nesting, and in addition shows both a polymorphism (indicated by the arrow) and a possible retroposition event (boxed).



We have succeeded in targeting PCR primers the HERV long terminal repeats and reverse transcriptase in various cancer cell lines and tumor tissues. A retroviral activity corresponding to different members of the two main HERV families was evidenced in cell lines and in tissues. No new integration site has been discovered yet but differences observed in normal versus tumor fingerprints and DNA array hybridizations are under further investigation (isolation and sequencing for identification).

Bubble-PCR, RDA, and SSH

As explained in the previous annual report, these methods originally proposed

were unsuccessful due to the apparent low frequency of novel retrovirus transposition and problems intrinsic to PCR, namely, insufficient specificity of PCR when primers are not exact matches for the targeted sequences. The background due to non-specific priming was simply too high for these methods to fully resolve new transposition events. Thus, we proposed to continue our work along the direction of using PAC arrays of genomic clones probed with retroviral flanking sequences to screen cancers for novel insertion events. As stated previously, "A new retroposition event will be accompanied by new, unique flanking sequences which should show up as hybridization to a new BAC or YAC clone". As it turns out, we used PAC arrays.

Recent progress

While we have not succeeded in demonstrating any novel insertional event of retroviral sequences in breast cancer, with our modified aims we have fully developed a method that should be able to detect novel insertional events should they occur for about half of the genome. The strategy works as follows. Probe is made from genomic DNA of normal-tumor pairs using an oligonucleotide homologous to the retroviral LTR (in this case, HERV). Two oligos were designed (LTR2 and LTR3) homologous to the two ends of the HERV LTR. These primers are directed toward the most highly conserved sequences in the LTR. The initial round of synthesis begins with one of these primers and extends from two positions due to the direct repeat nature of the LTR. The second strand synthesis is supported by *arbitrary priming*, where the same primer used for the first strand arbitrarily primes on the product from the first round. Thus, the final PCR amplifiable products have either LTR2 or LTR3 at both ends, depending on which was used. LTR2 and LTR3 are alternatives, and *not* used in the same synthesis reaction. Synthesis of the retroviral genome (i.e. between the LTRs) is inevitable. Second strand synthesis by arbitrary priming within the retroviral genome must happen only once in a while to guarantee that some retroviral sequence will be represented in the probe, and in fact it is predominant. Sequences outside of the retrovirus, per se, are amplified when the outward-facing primer is accompanied by an arbitrary priming event toward the retrovirus. These events capture unique sequence, and if a new retroviral insertion event occurs, this is where the novel, unique flanking sequence will be captured. Once a probe has been synthesized in the manner described, hybridization to arrays of PAC clones can, in principle, reveal new insertion events.

The PAC arrays we have used contain average 150 kb insertions, such that the entire mammalian genome can be contained in a minimal non-overlapping set of about 20,000. Since there are 10,000 HERVs in the genome, about half of these 20,000 contain retrovirus derived sequences. New insertion events into PAC-defined regions that already contain a retrovirus will be invisible to this approach because the probes contain large amounts of the retroviral genome internal to the LTRs. However, the other half of the genome can be probed because they do not contain retroviral genomic sequence. Improving matters somewhat are the facts that many of the older pro-retroviruses are diverged to the point that they are not detected by high stringency probing and that in many cases only a single LTR is found. In these cases, new retroviral insertion events will likely have higher homology to preexisting retroviral sequences, and when only a single LTR is present, only unique flanking sequence will be detectable by hybridization.

In one experiment, we wanted to calibrate the individual-to-individual variation which attends arbitrary priming. Figure 4 shows examples comparing two individuals using LTR2. There were very few differences (< 0.05%) commensurate with individual variation among humans. The differences probably arise mostly from differences in the arbitrary priming step due to sequence variation between individuals, but can also arise from differences in the positions of pro-retroviruses. This experiment shows that the

probe synthesis and hybridization methods are robust, and shows that we can detect a number of PACs roughly equivalent to the estimated 10,000 predicted to harbor pro-retroviruses.

Figure 4. Probes made using the same primers using DNA from two individuals.

a. First individual

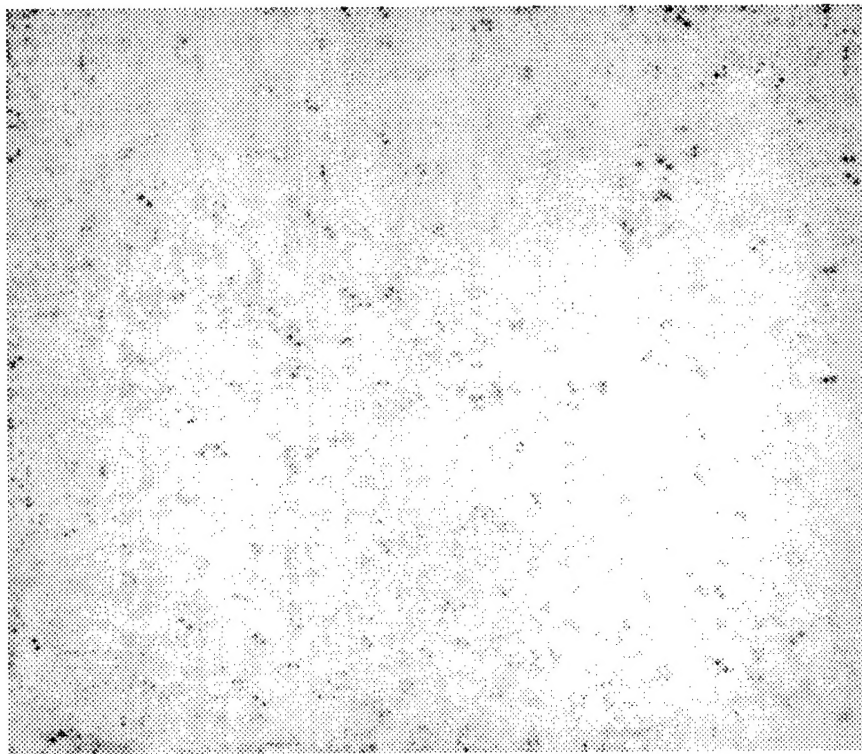
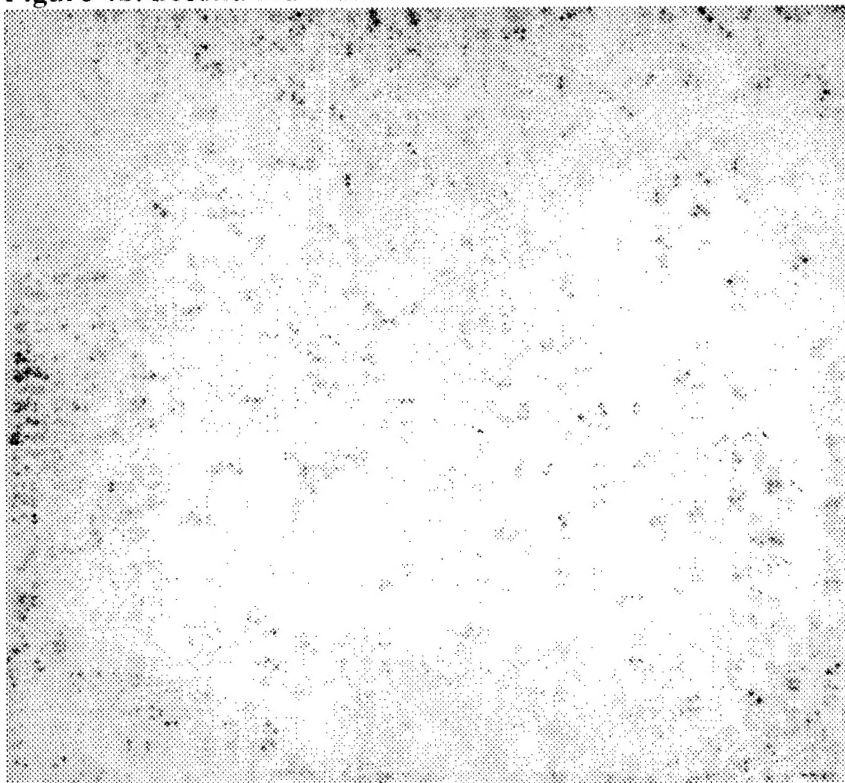


Figure 4b. Second individual.



Next, we compared the same normal DNA sample using either LTR2 or LTR3. It is predicted that the major source of difference between these two probes is due to the distribution of single LTRs. As mentioned above, complete pro-retroviruses contain the internal genomic sequences, and should be detected by probes derived from either LTR2 or LTR3. Isolated LTRs could also contribute probe from either primer, but sequence variation in isolated LTRs can result in detection by one and not the other, as can the presence or absence of an effective arbitrary priming site. Thus, in the experiment shown in **Figure 5**, many differences can be seen between hybridizations derived from the two oligos, and these differences should reflect, by and large, PACs that contain isolated LTRs. This difference is predicted to go away when a full length novel retroviral insertion event occurs. Because of the continuous genetic drift that has resulted in divergence of pro-retroviral insertions, and because it is difficult to know how efficiently arbitrary priming captures each pro-retrovirus, it is difficult to precisely estimate the number of pro-retroviruses and isolated LTRs. For recent novel insertional events, identical retroviral sequences would appear in more than one PAC, and these sequences would hybridize at high stringency. At high stringency, we observe signals from 10-20% of all PACs. This is a further improvement in the ability of the method to detect new insertion events over the >50% estimated above, because most of the endogenous pro-retroviral sequences will be unique and only able to hybridize to themselves. Presumably, those retroviruses still capable of moving will have retained certain essential features, such as promoter sequences, but the majority will fail in high stringency hybridization, leaving most PACs unhybridized. This, in turn, will make new insertion events into these PACs easier to detect. Finally, if differences on the order of two-fold can be detected using better technology, such as glass-based arrays, then the majority of PACs should become accessible.

In an experiment where normal and tumor DNAs were compared, no novel retroviral insertion events were identified. This is not surprising because the proposed mechanism, oncogenesis by retroviral insertion, is not expected to occur in all cancers. However, the result indicates that the method should be very robust against false positives. This experiment is shown in Figure 3.

Figure 5 a and b.: Probe derived from a. LTR2-specific and b. LTR2-specific.

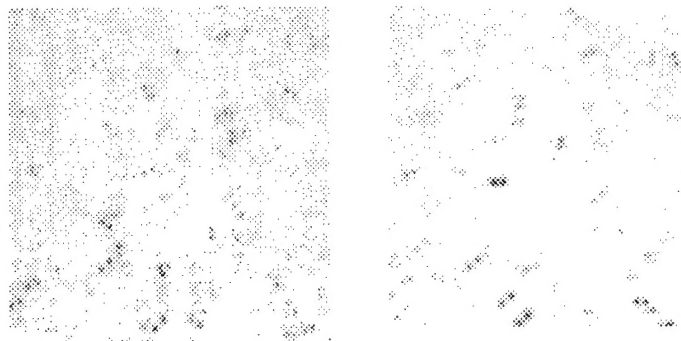


Figure 6a. Normal DNA

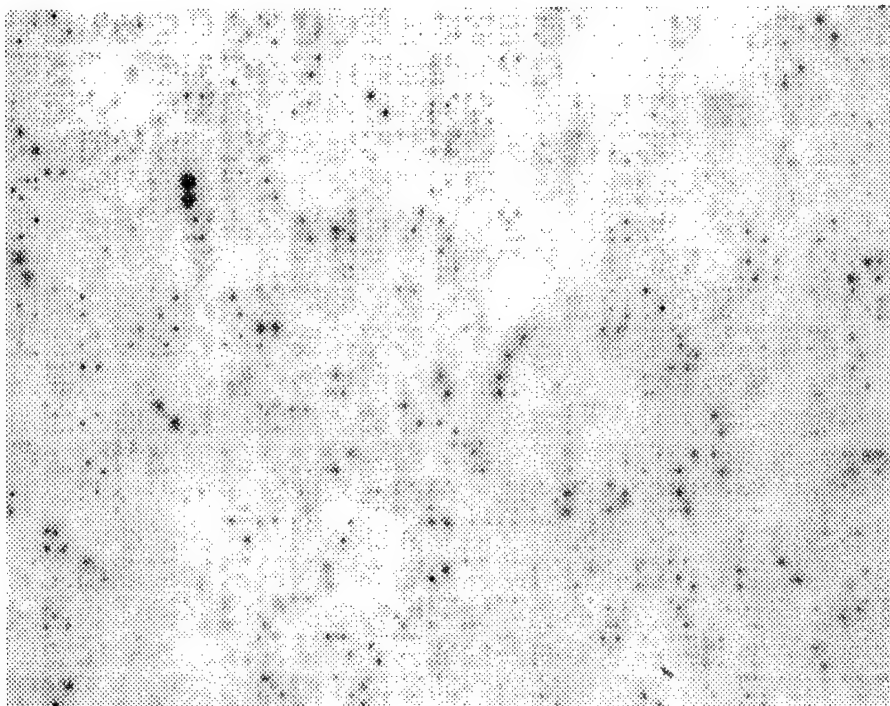
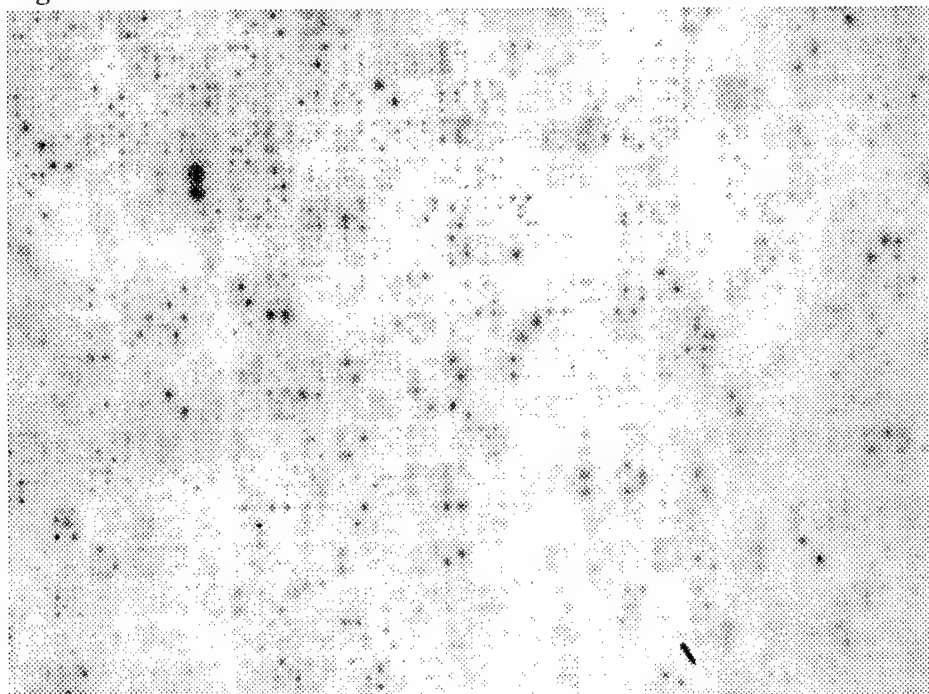


Figure 6b. Tumor DNA.



Close inspection of these figures reveals high reproducibility and no candidate retroinsertion events.

Conclusion:

The original strategies proposed for this project failed. Those strategies were based on "bubble PCR" in which retroviruses were intended to be specifically amplified from genomic DNA. This strategy fails because arbitrary priming events, while rare, are

favored by the enormous complexity of the human genome. Recognizing this problem, we devised the new strategy involving PAC arrays as described above. This new strategy can, in principle, detect more than half of all new retroviral insertion events. The hybridizations are based on the presence in the probe of unique flanking sequence around retroviruses. The retrovirus sequence itself contributes to background by hybridizing to all PACs that contain retrovirus sequences, and thus, only those insertion events in PACs that did not contain retrovirus sequences in the individual from which the PAC library was made are accessible to the method. In future experiments, however, one can envision blocking these "background" retroviral sequences with pure retroviral driver, such that the unique flanking sequence around novel insertion events could be detected. This would make the entire genome accessible to the method. Thus, we have partially succeeded in our original goal of devising a method that will allow the detection of retroviral sequences for about half of the genome, and have discerned a clear path toward completing development of the method.

Relevant publications

1. B Jung, T Vogt, F Mathieu-Daude, J Welsh, M McClelland, T Trenkle, C Weitzel, and F Kullmann (1998). Estrogen-responsive RING finger mRNA induction in gastrointestinal carcinoma cells following bile acid treatment. *Carcinogenesis*. 19:1901-1906.
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8. Pesole, G., Liuni, S., Grillo, G., Belichard, P., Trenkle, T., Welsh, J. and McClelland, M., (1998). GeneUp: A program to select short PCR primer pairs that occur in multiple members of sequence lists. *BioTechniques* 25; 112-117.
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10. T Vogt, W Stolz, J Welsh, B Jung, RS Kerbel, H Kobayashi, M Landthaler, and M McClelland (1998). Overexpression of Lerk-5/Eplg5 messenger RNA: a novel marker for increased tumorigenicity and metastatic potential in human malignant melanomas. *Clin. Cancer. Res.* 4:791-797.
11. Vogt, T., Mathieu-Daudé, F., Kullmann, F., Welsh, J. and McClelland, M. (1997) Fingerprinting of DNA and RNA using arbitrarily primed PCR. In: *DNA Markers:*

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1. B Jung, T Vogt, F Mathieu-Daude, J Welsh, M McClelland, T Trenkle, C Weitzel, and F Kullmann (1998). Estrogen-responsive RING finger mRNA induction in gastrointestinal carcinoma cells following bile acid treatment. *Carcinogenesis* 19:1901-1906.
2. Pesole, G., Liuni, S., Grillo, G., Belichard, P., Trenkle, T., Welsh, J. and McClelland, M., (1998). GeneUp: A program to select short PCR primer pairs that occur in multiple members of sequence lists. *BioTechniques* 25; 112-117.
3. T Trenkle, J Welsh, B Jung, F Mathieu-Daude, and M McClelland (1998). Non-stoichiometric reduced complexity probes for cDNA arrays. *Nucleic Acids Res.* 26:3883-3891.
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PERSONNEL REPORT

Name	Percent of Effort	Date(s)
John Welsh	10%	10/01/97-02/28/98
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" "	53%	09/01/99-09/21/99
Mami Hayakawa	100%	01/01/99-09/21/99

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